Transformation and isolation of allelic exchange mutants of *Chlamydia psittaci* using recombinant DNA introduced by electroporation

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To facilitate genetic investigations in the obligate intracellular pathogens *Chlamydia*, the ability to construct variants by homologous recombination was investigated in *C. psittaci* 6BC. The single rRNA operon was targeted with a synthetic 16S rRNA allele, harboring three nucleotide substitutions over 398 bp, which imparts resistance to kasugamycin (Ksm) and spectinomycin (Spc) and causes loss of one HpaI restriction site. A fourth, silent mutation was introduced 654 bp downstream in the beginning of the 23S rRNA gene. *C. psittaci* 6BC infectious particles were electroporated with various concentrations of circular or linearized plasmids containing different lengths of the rRNA region homologous to the chromosomal copy except for the four nucleotide substitutions. Ksm and Spc were added 18 h after inoculation onto confluent cell monolayers in the plaque assay. Resistant plaques were picked and expanded with selection 10 days later before collecting DNA for analysis by PCR, restriction mapping, sequencing, or Southern. Spontaneous resistance to Ksm and Spc was never observed in mock electroporated bacteria (frequency <6.2×10^{-9}). Conversely, double resistance and replacement of the 16S rRNA gene were observed when *C. psittaci* was electroporated with the recombination substrates. Highest efficiency was obtained with 10 μg of circular vector prepared in a DNA methylase-deficient *Escherichia coli* ($1.9 \pm 1.1 \times 10^{-6}$, $n = 7$). Coinheritance of the silent 23S rRNA mutation was seen in 46 of 67 recombinants analyzed, illustrating DNA exchange of up to 1,052 bp in length. These findings provide the first step toward genetic manipulation of *Chlamydia*.

recombination | selection | plaque assay

Chlamydiae are obligate intracellular bacteria that cause a wide variety of infectious diseases in humans and animals (1). *C. trachomatis* infections may be asymptomatic, but chronic inflammation can lead to blindness in cases of trachoma and pelvic inflammatory disease, and infertility in cases of urogenital infections. *C. psittaci* is the agent of avian psittacosis, but it can also cause severe pulmonary infections in humans. *C. psittaci* has been isolated from a wide range of wild and domesticated birds where it can produce asymptomatic or symptomatic infections with a systemic and occasionally fatal outcome. Transmission to humans results mainly in influenza-like illness, although severe pneumonia, endocarditis, myocarditis, hepatitis, and encephalitis have been reported (2).

The ability to specifically inactivate a gene and restore the inactivated gene is central to show gene function and fulfill molecular Koch's postulates for microbial pathogenicity (3). The complete sequencing of 11 strains of *Chlamydia* in the past 10 years and the rapid expansion of genomic, comparative genomic, transcriptomic, and proteomic analysis of these pathogens have created a collection of hypotheses that need to be tested through classic genetic mutation and complementation studies. However, the particular physiology of these organisms poses serious obstacles in generating the tools needed to perform genetic analysis and define the genes that are important to the biology, pathogenicity, or transmission of *Chlamydia* (4). Besides long generation times and the requirement for growth within permissive cells, the developmental cycle of *Chlamydia* is biphasic, alternating between small, infectious, extracellular forms, the elementary bodies (EBs), and larger, noninfectious, intracellular, replicative forms, the reticulate bodies (RBs). Two to 4 hours following uptake by a host cell, EBs convert to RBs within a membrane-bound vacuole termed an inclusion. The RBs replicate by binary fission and then, 18–48 h postinfection (p.i.), depending on the species, start to redifferentiate back to EBs and exit the cell to repeat the cycle (5).

To realize the full potential of genetic analysis of *Chlamydia*, it would be useful to have a means of transforming these pathogens using circular plasmids that can be easily manipulated in *Escherichia coli*. Almost 15 years ago, Tam *et al.* (6) reported transient transformation of *C. trachomatis* following electroporation of EBs with recombinant plasmid DNA derived from the native chlamydial cryptic plasmid. We sought to develop a stable transformation system involving homologous gene targeting, as this would allow directed mutation of the genome as well as long-term expression of introduced DNA. Such allelic replacement technology has proven extremely valuable in numerous species to selectively alter genes of interest in the host chromosome (7, 8).

An ideal target for testing the feasibility of mutagenesis by homologous recombination in *C. psittaci* is its single-copy rRNA operon. We recently showed that specific mutations in the 16S rRNA or 23S rRNA genes are associated with resistance to spectinomycin (Spc) or to the clinically relevant macrolides, respectively (9, 10). Here we constructed, in *E. coli*, pUC plasmid derivatives carrying 8.1-, 6.5-, 5.5-, 4.8-, or 2.7-kb rRNA regions of *C. psittaci* 6BC, harboring 2 antibiotic resistance mutations plus 2 unselected mutations, within a 1,052-bp segment. After introduction by electroporation in the wild-type strain, we selected for allele replacement of the endogenous rRNA operon with the mutated copy from the plasmid by homologous recombination. The findings of this study provide a solid foundation toward developing DNA integration and genetic analysis tools for *Chlamydia*.

Results

Selection for an Aminoglycoside-Resistant Double Mutant of *C. psittaci* 6BC. Aminoglycoside antibiotics inhibit protein translation by interacting with the 16S rRNA of the 30S ribosomal subunit. Due to poor permeability across the plasma membrane of eukaryotic cells, these antibiotics are not used for the treatment of chlamydial infections. However, we showed previously that *C. psittaci* 6BC is

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Table 1. Frequencies of spontaneous mutation to kasugamycin and/or spectinomycin resistance in *C. psittaci* 6BC

Strains	Drug	Concentration, mg/ml	Frequency of resistance	Nucleotide change in the 16S rRNA gene*
BC_{RB}†	Spc‡	0.3	1.1×10^{-6}‡	$C_{1192}U$, $A_{1191}G$, $C_{1192}G$, $G_{1193}C$‡
	Ksm	2; 5	2.3×10^{-5}; 1.3×10^{-5}	$A_{794}G$
	Spc + Ksm	0.3; 2	$< 5 \times 10^{-9}$	NA
BC0E1 (Spc^R)	Spc + Ksm	0.3; 2	2×10^{-6}	$C_{1192}U$ (Spc^R) + $A_{794}G$ (Ksm^R)

The frequency of mutation to drug resistance was determined by dividing the number of PFU on medium with drug by the number of PFU added to the monolayer (as measured by PFU titer in the absence of drug) (9, 10). See Table S1 for strains. NA, not applicable

*E. coli numbering.

†Clonal wild-type *C. psittaci* 6BC strain described in ref. 10.

‡Data from ref. 9.

inhibited *in vitro*, in the plaque assay, by the aminoglycoside Spc. We obtained Spc^R mutants at a frequency of $\approx 1 \times 10^{-6}$. These mutants resulted from single-nucleotide changes in the unique 16S rRNA gene at position 1191, 1192, or 1193 (9). (Note: We use the *E. coli* numbering system throughout when referring to nucleotide(s) in rRNA genes.) We examined the *in vitro* sensitivity of *C. psittaci* 6BC for 8 additional aminoglycosides (see *Materials and Methods*) and found that, although streptomycin, tobramycin, and paromomycin were slightly inhibitory to the bacteria's ability to grow in L2 mouse fibroblast cells, only kasugamycin (Ksm) was efficient enough to inhibit plaque formation of 10^5 plaque-forming units (PFU) in those cells, with a minimum inhibitory concentration of 1.1 mg/mL. Spontaneous Ksm^R mutants were isolated for the wild-type *C. psittaci* 6BC strain and the Spc^R BC0E1 variant (9) with frequencies of 2.3×10^{-5} and 2.0×10^{-6} respectively (Table 1). Analyses of the 16S rRNA gene from 24 independent Ksm^R variants revealed the same $A_{794}G$ mutation previously isolated in a Ksm^R strain of *E. coli* expressing a single rRNA operon (11). When Ksm resistance was selected in BC0E1, both the Ksm^R $A_{794}G$ mutation and the original Spc^R $C_{1192}T$ mutation were identified in the 16S rRNA gene, indicating no antagonism between the 2 resistance mutations. No plaques were recovered when up to 10^8 wild-type parental cells were infected in the plaque assay in the presence of both Spc and Ksm (Table 1). This suggests that spontaneous mutations to dual resistance occur at an undetectable frequency. Consequently, we chose the rRNA operon from the Ksm^R Spc^R *C. psittaci* 6BC BCKS1 isolate [supporting information (SI) Table S1] as the source for the selective marker for allelic exchange.

Construction of the rRNA Operon-Based Selection Vector for *C. psittaci*. pRAK407 (Fig. 1) was created from 6 different PCR amplifications, clonings, and sequencings and 5 subclonings (Table S1). It is a pUC derivative carrying the chloramphenicol resistance *cat* gene for selection in *E. coli* and the double Ksm Spc-resistant 16S allele of *C. psittaci* 6BC together with 3 kb DNA upstream and 3.5 kb downstream from the mutated 16S rRNA gene. An additional mutation, A to G, was created during the construction of pRAK407 at position 72 in the beginning of the 23S rRNA gene, 654 bp downstream of the Spc^R mutation. Although this nucleotide is highly conserved in *Chlamydia* and in *E. coli* (Fig. S1*A*), its importance in bacterial physiology has not been characterized.

Sequence alignment of the 16S rRNA genes available in GenBank for 42 different strains of *C. psittaci* revealed a natural heterogeneity in the nucleotide at position 1071: 25 strains, including strain 6BC, harbor a C, whereas the remaining 17 possess a T (Fig. S1*B*). We introduced the $C_{1071}T$ mutation into the 16S Ksm Spc-resistant *C. psittaci* 6BC allele to create pRAK426 (Fig. 1). This mutation is not expected to affect the bacterial physiology once introduced into *C. psittaci* 6BC; however, it disrupts the HpaI $GTTAAC_{1071}$ restriction site and thus provides a powerful tool to analyze the recombinants.

Optimization of Electroporation for *C. psittaci* 6BC. All genetic systems for the disruption of chromosomal loci depend on the ability to deliver DNA into the bacterial cell. Consequently, we sought to optimize electroporation conditions to introduce DNA into EBs, the small ($\approx$0.3 μm in diameter), environmentally stable chlamydial infectious particles with a condensed nucleoid structure protected by a rigid cell wall. PCR (data not shown) and comparative qPCR (Table 2) analyses show that a foreign marker became protected from extracellular DNase digestion after electroporation with *C. psittaci* 6BC EBs. We found that the amount of DNA

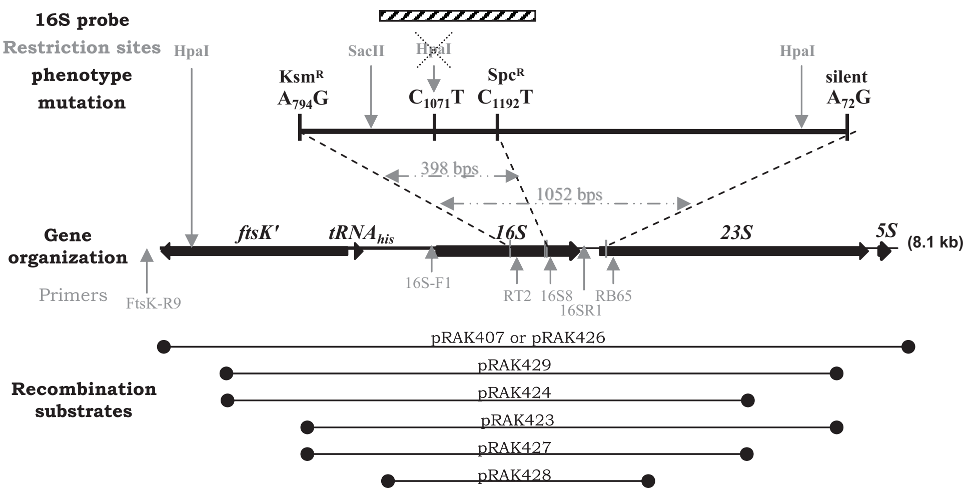

Fig. 1. Gene organization of *C. psittaci* 6BC rRNA region inserted in the recombination substrates. Analytical primers (Table S2); SacII and HpaI restriction sites; and the 16S probe used in Fig. 2*B* and Fig. S3 are shown.

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Table 2. Influence of electroporation parameters on the survival of *C. psittaci* and the protection of foreign DNA from extracellular DNase I digestion

Resistance, Ω	Field strength, kV/cm*	Number of pulses	Time constant, ms	Viable EBs, PFU†	$-\Delta\Delta$Ct‡
-	-	0	-	$7.6 \pm 0.0 \times 10^7$	0
200	18	1	4.5	$7.2 \pm 0.6 \times 10^7$	5.1
200	18	2	2×4.5	$6.1 \pm 0.5 \times 10^7$	6.2 ± 0.3
400	12.5	1	8.8	$6.5 \pm 0.5 \times 10^7$	6.4 ± 0.3
400	12.5	2	2×8.8	$5.5 \pm 0.2 \times 10^7$	8.1 ± 0.3
600	16	1	12.6	$5.8 \pm 0.3 \times 10^7$	9.3 ± 0.2
600	16	2	2×12.6	$3.4 \pm 0.3 \times 10^7$	10.0 ± 0.3

*Capacitance was always set to 25 μF.

†Following electroporation, EBs were resuspended in PBS supplemented with 10 mM $MgCl_2$. Dilutions of the bacterial suspensions were infected in the plaque assay for enumeration of viable bacteria. Values are means ± SD of duplicate samples.

‡qPCR analysis of a plasmid-encoded foreign gene (*aadA*) after electroporation and 4 cycles of 15-min DNase I treatment at 37°C, normalized to the amount of *C. psittaci* 16S rRNA gene and relative to the amount obtained in the absence of electroporation. The higher the $-\Delta\Delta$Ct value, the higher the amount of vector present in the sample. Values are means ± SD of triplicate samples.

surviving DNase treatment increased when higher time constants were obtained, being 2^{10}-fold (≈1,000-fold) greater, with 2 pulses of 12.8 ms each, than in the absence of electroporation (Table 2). Because longer exposure to high-voltage fields resulted in greater DNA introduction, we used 2 pulses at 1.6 kV, 600 Ω, and 25 μF in all chlamydial electroporation assays. These conditions reduced bacteria viability by only ≈2-fold (Table 2).

Isolation and Molecular Analysis of the Double Spc^R Ksm^R *C. psittaci* Recombinants. Initial experiments used pRAK407 and pRAK426 DNA to transform *C. psittaci*. Following electroporation, *C. psittaci* 6BC EBs were diluted in 1× Dulbecco's Modified Eagle Medium (DMEM; GIBCO) and used to infect confluent monolayers of L2 mouse fibroblasts in the plaque assay (9). Both 300 μg/mL Spc and 3 mg/mL Ksm were added at 18 h p.i., before RBs started conversion back to EBs (12). We observed that the monolayer was able to tolerate infection with 10^7 EBs under such conditions, corresponding to a multiplicity of infection of 1. Between 10 and 14 days p.i., resistant plaques were picked and expanded again in the plaque assay with drug selection at 2 h p.i. Total DNA was prepared from infected cells when plaques started to appear, or at 10 days p.i., and further analyzed (Fig. S2). The chromosomal 16S rRNA gene and its upstream region were specifically amplified from a total of 329 individual Ksm^R Spc^R plaques that expanded with selection, using the 16S-R1 and the FtsK-R9 primers (Fig. 1 and Table S2). FtsK-R9 anneals to *ftsK* in a region that is not included in the transformation allele. This strategy precludes amplification of episomal or illegitimately integrated plasmids and allows only the amplification of DNA from the chromosomal rRNA locus. Sequencing of the 16S rRNA gene in 37 recombinants showed the presence of the 2 resistance mutations at position 794 and 1192, as expected from the dual-resistance phenotype. HpaI restriction mapping in each 16S rRNA region PCR product from 329 recombinants derived from pRAK426 revealed the absence of a 415-bp fragment (Figs. 1 and 2*A*), indicating the coinheritance of the $C_{1071}T$ mutation resulting in the disruption of one HpaI restriction site in the chromosomal 16S rRNA allele. Southern blot analysis of highly purified genomic DNA from the parent BC_{RB} and 4 independent pRAK426-derived recombinants also confirmed the lack of one HpaI site in the recombinant 16S rRNA gene (Fig. 2*B*). The 18 recombinants derived from pRAK407 showed conservation of the HpaI site (e.g., Fig. 2*A*, lane 12). The presence of only a single SacII fragment in the recombinants also excludes the possibility that

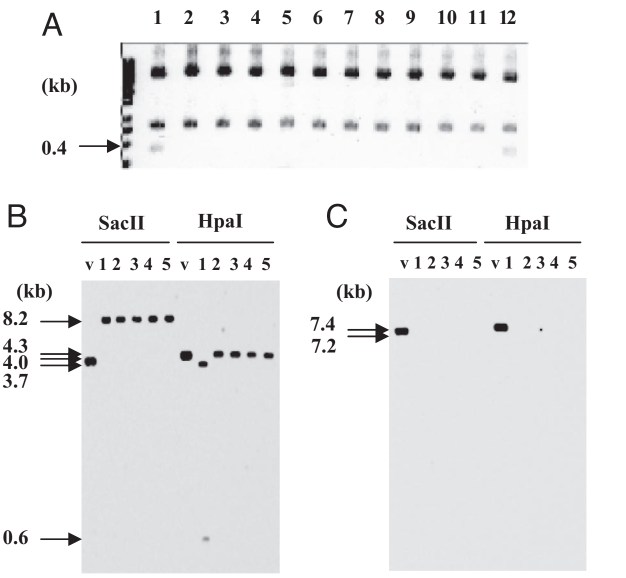

Fig. 2. PCR and Southern hybridization analyses of *C. psittaci* 6BC recombinants. Allelic replacement of the native 16S rRNA gene by the recombinant 16S rRNA gene present in pRAK426 and its derivatives yields altered restriction profile after digestion with the restriction enzyme HpaI. (*A*) HpaI restriction mapping of PCR amplified *C. psittaci* 6BC chromosomal 16S rRNA and upstream region. HpaI digestion of the 4,962-bp region in the wild-type strain (lane 1) or in recombinants obtained using pRAK407 (lane 12) shows 3 bands (i.e., 3,738 bp, 809 bp, and 415 bp), whereas recombinants obtained using pRAK426 and its derivatives (lanes 2–11) that contain the $C_{1071}T$ mutation show only 2 bands (i.e., 4,153 bp and 809 bp). Gene Ruler 1kb DNA Ladder (MBI Fermentas). (*B*) Southern hybridization of *C. psittaci* 6BC genomic DNA using the 16S probe shown in Fig. 1. This probe recognizes a 4.0-kb SacII fragment and a 4.3-kb HpaI fragment in the recombination plasmid pRAK426 (lanes v). One microgram of DNA collected from RenoCal-76 purified EBs from the wild-type parent (lane 1) and 4 independent pRAK426-derived recombinants (lanes 2–5) was digested with SacII or HpaI. The probe binding pattern differed between the parent and the recombinants when the genomic DNA was digested with HpaI, shifting from 2 bands at 0.6 and 3.7 kb to one 4.3-kb band in the recombinants. This shows that the recombinants have replaced the wild-type 16S rRNA allele harboring an internal HpaI site with the recombinant allele from pRAK426 that lacks the HpaI site. However, the 16S probe annealed to a single 8.2-kb SacII fragment in all 5 *C. psittaci* 6BC isolates, including the 4 recombinants showing that the recombination plasmid was not maintained and did not integrate in the bacterial genome. In addition, the homologous recombination event did not affect the immediate surroundings at the 3′ end of the chromosomal 16S rRNA gene. (*C*) Southern hybridization of *C. psittaci* 6BC genomic DNA using the *cat* probe. This probe annealed to a 7.4-kb SacII fragment and a 7.2-kb HpaI fragment in the recombination plasmid pRAK426 (lanes v). No hybridization signal was detected in the 5 *C. psittaci* 6BC isolates. (B) and (C) show the same membrane, which was stripped and reprobed.

the transformation plasmid had integrated into the chromosome, because such an event would have produced multiple hybridizing bands. For 328 recombinants analyzed, the phenotypes imparted by the 3 marker nucleotides at positions 794, 1071, and 1192 in the 16S rRNA gene indicated that DNA replacement occurred over a minimum of 398 bps.

Relationship between Recombination Frequency and Physical State of the Recombination Substrate. In a typical transformation experiment starting with 10^7 *C. psittaci* EBs, ≈5×10^6 PFU survived electroporation. Transformation with a minimum of 10 μg of circular or NotI-linearized plasmid that separated the chlamydial rRNA region from the vector backbone produced recombinants at a frequency ≈10^{-6} (Table S3 and Table 3). However, the assay was much less reproducible when linear DNA was used, because electroporation was frequently accompanied by arcing or a poor time constant, resulting in elevated bacterial killing. Consequently,

Table 3. Relationship between recombination frequency and length of homology on the unmethylated circular recombination donor plasmid DNA

Recombination plasmid	Length of homology upsteam to the Ksm^R mutation // downsteam to the Spc^R (or 23S rRNA) mutation	Amount of electroporated DNA, μg	Frequency of recombination	No. of Ksm^R Spc^R recombinants per transformation	Distribution of the $A_{72}G$ mutation in the chromosomal inherited 23S rRNA gene*: A_{72}	G_{72}
pRAK426	3.8 Kb // 3.9 (or 3.2) Kb	20	$1.8 \pm 0.7 \times 10^{-6}$, $n = 7$	4, 16, 12, 12, 16, 10, 10	6	27
		10	$1.9 \pm 1.1 \times 10^{-6}$, $n = 7$	7, 12, 14, 4, 6, 3, 3		
pRAK429	3.0 Kb // 3.1 (or 2.5) Kb	20	$0.7 \pm 0.5 \times 10^{-6}$, $n = 3$	2, 6, 2, 10, 4	-	6
		10	$1.1 \pm 0.4 \times 10^{-6}$, $n = 3$	6, 4, 6, 10, 5		
pRAK423	2.0 Kb // 3.1 (or 2.5) Kb	20	$1.2 \pm 0.6 \times 10^{-6}$, $n = 2$	12, 11	10	7†
		10	0.4×10^{-6}, $n = 1$	3		
pRAK424	3.0 Kb // 2.25 (or 1.6) Kb	20	1.0×10^{-6}, $n = 1$	7	2	6
		10	0.6×10^{-6}, $n = 1$	2		
pRAK427	2.16 Kb // 2.25 (or 1.6) Kb	20	$0.6 \pm 0.8 \times 10^{-6}$, $n = 2$	3, 6	2	-
		10	$< 0.36 \times 10^{-6}$, $n = 1$	0		
pRAK428	1.27 Kb // 1.0 (or 0.4) Kb	20	$0.3 \pm 0.4 \times 10^{-6}$, $n = 2$	6, 0	1	-
		10	$< 0.36 \times 10^{-6}$, $n = 1$	0		

*In randomly selected recombinants.
†One recombinant did not acquire the $C_{1071}T$ mutation in the chromosomal 16S rRNA gene (see *Discussion*).

we never recovered more than 2 resistant plaques per transformation with NotI-linearized plasmid (Table S3).

The DNA of *E. coli* K-12 contains modified bases (6-methyladenine and 5-methylcytosine) resulting from the action of 3 DNA methyltransferases, EcoK (encoded by the *hsd* host specificity genes), Dam, and Dcm (13). Although analysis of the sequences available for 10 different chlamydial genomes yielded ORFs with predicted methyltransferase motifs, none appeared directed against DNA, suggesting that DNA in *Chlamydia* is not modified. Restriction analysis of the native chlamydial plasmid using methylation-sensitive enzymes showed that *C. trachomatis* L2 DNA is not methylated *in vivo* (data not shown). The recombination donor plasmid prepared in an *E. coli* host that was mutated in all 3 DNA methylase genes—*hsdS*, *dcm*, and *dam*—was a slightly better substrate for recombination, as we obtained up to 14 recombinants per transformation with 10 or 20 μg of circular unmethylated plasmid, for a recombination frequency close to 3.0×10^{-6} (Table S3 and Table 3).

Defining the Minimal Length of Sequence Homology Required for Homologous Recombination in *C. psittaci* 6BC. To determine the minimum size of DNA necessary for homologous recombination in *C. psittaci* 6BC, we constructed a set of derivatives from the initial pRAK426 construct harboring various deletions 5′ and/or 3′ to the 16S rRNA double-resistance allele (Fig. 1). The recombination frequency, which is dependant on both the number of recombinants and the number of EBs that survived electroporation, varied from the limit of detection ($\approx 0.4 \times 10^{-6}$) to $\approx 2 \times 10^{-6}$, depending on the construct. We saw a drop in the number of recombinants obtained when the flanking regions were reduced to <2.0 kb of strict homology, although higher amounts of unmethylated plasmid could compensate for shorter lengths of homology to some extent (Table 3).

The A-to-G single nucleotide polymorphysm (SNP) located in the recombination plasmid at position 72 of the 23S rRNA gene allowed us to extend our analysis on the length of DNA that recombined into the *C. psittaci* 6BC genome. This SNP lying 1,052 and 654 bps downstream to the Ksm^R and the Spc^R mutations, respectively, was coinherited with both 16S rRNA resistance mutations in 46 of 67 independent recombinants, showing DNA exchange over a region up to 1,052 bps in length. Acquisition of this 23S rRNA mutation seemed also dependant on the size of homology flanking the nucleotide mutation. Nonetheless, there was 75% coinheritance with only 1.6 kb of flanking DNA (Table 3). One recombinant from pRAK423 carried the $A_{72}G$ mutation in the 23S rRNA, but showed in its 16S rRNA gene only the 2 antibiotic-resistant mutations and not the intervening $C_{1071}T$ allele. This finding was unexpected and probably due to gene conversion (see *Discussion*).

Fate of the Circular Recombination Substrate. The hybridization analysis of highly purified genomic DNA did not reveal the presence of multiple 16S rRNA gene copies in the recombinants (Fig. 2*B*), nor were we able to detect the recombination vector using a probe directed against the *cat* gene (Fig. 2*C*). This indicates that the recombination substrate was not maintained and did not integrate into the recombinant chromosome. However, ectopic expression of the Ksm^R Spc^R 16S rRNA allele could in theory confer some level of resistance to *C. psittaci*, because expression of resistance linked to mutated rRNA genes requires that a majority of the cellular rRNA be of the mutant form (14). Three recombinants of the 329 obtained still showed the presence of the pUC derivative by PCR amplification of the *cat* marker in infected cell lysates prepared 18 days posttransformation (data not shown), and we were able to recover the recombination plasmid intact by transformation into *E. coli* EC100. Yet sequence analysis of the chromosomal ribosomal operon did not show the presence of the parental 16S rRNA allele. Southern blot hybridization of 2 of the recombinants using the *cat* probe and the 16S probes confirmed that the recombination plasmid was indeed episomal and not integrated somewhere in the bacterial chromosome (Fig. S3). Serial dilution of the third recombinant and additional passage in cell culture in the presence of drug resulted in loss of the plasmid 25 days posttransformation, illustrating the instability of the pUC replicon in *C. psittaci* 6BC.

Stability of the Allelic Exchange in *C. psittaci* 6BC Recombinants. Six independent recombinants, including 3 with the 23S rRNA $A_{72}G$ SNP, were purified in the plaque assay 11 days posttransformation and then expanded with drug selection for 6 additional days (Fig. S2). All 6 gave similar titers in the plaque assay in the presence or absence of selection. Plaques were further grown for 8 days in the absence of selection, then picked and expanded in the absence of selection for 6 more days (Fig. S2). The quantity of EBs contained in each final culture was similar in the presence or absence of selection, confirming the stability of the Ksm^R and Spc^R phenotypes. We then purified and expanded 8 plaques that developed in the absence of selection for each of the 6 recombinants for DNA analysis (Fig. S2). The nucleotides at positions 794, 1071, and 1192

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in the 16S rRNA gene and 72 in the 23S rRNA gene were identical to those seen in the original recombinant, showing that the DNA exchange between the recombination vector and the bacterial genome led to stable transformation of *C. psittaci* 6BC.

Discussion

Progress in exploiting chlamydial genome information has been severely impeded by the complete lack of genetic tools for the directed manipulation of specific genes in these obligate intracellular Gram-negative pathogens (4). Although interstrain, intergenic, and intragenic recombination has been proposed to generate sequence variations in chlamydiae (15, 16), this study proves that recombinant DNA can be introduced into *C. psittaci* 6BC and promote recombination/exchange of "heterologous" DNA sequences into the bacterial chromosome.

Our findings show that a high electric field (i.e., 16 kV/cm) for ≈13 ms was optimal for DNA introduction into *C. psittaci* 6BC (Table 2). Similar conditions were optimal for *C. trachomatis* L2 (data not shown). Small cells such as chlamydial EBs generally require high electric fields for successful transformation, and similar conditions have been reported to be efficient for *Coxiella burnetii* transformation (17). Tam *et al.* (6) used 10.0 kV/cm, 400 Ω, and 25 μF in their electroporation protocol but reported poor postelectroporation recovery for *C. trachomatis* L2. In our study, viability of *C. psittaci* 6BC (Table 2) or *C. trachomatis* L2 (data not shown) was reduced by less than 3-fold as long as both DNA and EBs were resuspended in water without salt or other impurities. Consequently, we propose that the main factor responsible for the higher bacterial killing observed by Tam *et al.* (6) was probably the use of an electroporation buffer composed of sucrose, Hepes, and glycerol.

A challenge in the development of genetic tools for *Chlamydia* is that only a few antibiotic resistance determinants can be used. Deliberate introduction of genes conferring resistance to drugs used to treat chlamydial infections, such as tetracyclines, macrolides, fluoroquinolones, or amoxicillin (18), is restricted. The obligate intracellular lifestyle of these pathogens poses an additional challenge because an efficient antichlamydial drug must cross 4 permeability barriers (i.e., eukaryotic, inclusion, and outer and inner membranes) to reach the bacterial target. Although aminoglycosides are considered clinically inactive against obligate intracellular bacteria due to their low level of intracellular penetration, we found that both Spc and Ksm were inhibitory for *C. psittaci* in the plaque assay. We isolated spontaneous drug-resistant variants due to specific mutations in the unique 16S rRNA gene. Because expression of aminoglycoside resistance requires that >50% of the ribosome population be of the resistant phenotype, similar mutations conferring resistance to Spc or Ksm could not be isolated in *C. trachomatis* L2, which harbors 2 rRNA operons (9).

The transformation/recombination system described in this study allows us to introduce desired mutations into the single-copy rRNA gene of *C. psittaci* 6BC using a plasmid that contains a fragment of *C. psittaci* DNA containing the entire rRNA operon, including the selective markers in the 16S rRNA gene, which serves as the recombination substrate. The pUC backbone permits high-copy vector replication in *E. coli* and renders possible the application of standard genetic engineering and *in vitro* mutagenesis techniques. ColE1-type plasmids such as pUC are narrow host-range plasmids that replicate stably only in enterobacteriaceae (19). Consequently, they should behave like a suicide plasmid in *Chlamydia*, helping to promote selection of recombinants (20). We were still able to detect the recombinant vector, in its original configuration, in 3 recombinants at 18 days p.i., and, in at least 1 recombinant, the plasmid was still detected in infected cells at 25 days posttransformation. Although this represents less than 1% of *C. psittaci* recombinants, these data suggest a basic level of replication/maintenance in *C. psittaci* 6BC, similar to *Legionella pneumophila* in which ColE1 plasmids can be maintained under antibiotic selection, with low-copy number and instability (21). In our study, selection of transformants was applied only at 18 h p.i., corresponding to about 10 bacterial generations (12), and would not be sufficient to maintain extrachromosomal plasmids once the marker has recombined into the chromosome. Additional studies are thus necessary to confirm possible replication and maintenance of pUC vectors in *Chlamydia*.

Several features of our transformation strategy allow us a high degree of confidence in concluding that we recovered genetically modified, recombinant *C. psittaci* 6BC. (*i*) Because Spc and Ksm resistance are recessive in a merodiploid strain, expression of the resistance phenotype requires elimination of the wild-type gene (by gene replacement). This constraint precludes selection of events in which the circular vector would have integrated within the target sequence by single-crossover homologous recombination. Our analysis shows that the only cells that survive under the selective growth conditions were those in which the mutated region in the plasmid borne rRNA operon replaced the corresponding region of the chromosomal rRNA operon. This plasmid-to-chromosome allelic exchange is accompanied by loss of the plasmid vector and results in the transformed cells having a single copy of the rRNA operon carrying the selected mutations. (*ii*) Two nucleotide substitutions, 398 bp apart, are present in the *C. psittaci* 16S rRNA gene on the recombinant plasmids to promote resistance to the 2 ribosomal drugs Ksm and Spc. The use of 2 antibiotic-resistance markers in the vector is essential for selecting authentic transformants and for distinguishing them from single spontaneous point mutants. The latter appear with high frequencies when selected on either Ksm ($\approx 10^{-5}$) or Spc ($\approx 10^{-6}$). However, because 2 independent mutations must occur spontaneously within the same cell to render it resistant to both antibiotics, the frequency of such events should be vanishingly low ($\approx 10^{-11}$) such that only true transformants would grow in the presence of both drugs. (*iii*) The incorporation of 2 additional nucleotide changes that have never been described in *C. psittaci* 6BC into our recombination substrate further excluded the possibility for spontaneous emergence of a mutant harboring the Spc^R and Ksm^R alleles, as well as these 2 unselected alleles.

Recombinants were obtained at frequencies greater than 10^{-6}. Efficiency of homologous recombination was dependent on the size of the homologous DNA segments in the plasmid and chromosome. When the plasmid borne rRNA-encoding segment was reduced from 8 kb to 2.5 kb, the efficiency of transformation/recombination dropped almost 10-fold. The exact size of the plasmid DNA fragment that recombines into the chromosome is unknown, but it lies between a minimum of 0.4 kb (the distance between the 2 Ksm^R and Spc^R markers) to a maximum confirmed size of 1 kb (the distance between the Ksm^R marker and the unselected mutation in the 23S rRNA gene). However, the size could formally be as large as 8 kb, the largest plasmid-borne rRNA fragment used in this study.

Homologous recombination has a central role in the repair of DNA double-strand breaks, interstrand crosslinks, and collapsed replication forks and involves the exchange of strands between 2 homologous DNA molecules catalyzed by the RecA family of ATPases, conserved from bacteria to humans (22, 23). The exact mechanism of sequence replacement observed in this study is not known as it could involve reciprocal recombination via 2 successive crossovers (i.e., true allelic exchange) or nonreciprocal recombination via gene conversion (i.e., allelic replacement) that does not always involve crossovers but does require DNA synthesis. In 1 recombinant, we did not see transfer of the mutation located in between the 2 resistance mutations to the bacterial chromosome. Similar recombinants, displaying 2 tracts of continuous gene replacement with a marker or two between these tracts that did not show sequence replacement, have been previously observed in *Rhizobium etli* (24). It was speculated that rather than emerging from a rare quadruple crossover, these discontinuous sequence transfers more likely resulted from gene conversion.

In this study, we selected for the occurrence of homologous recombination events with the intent of developing methods for

DNA integration, gene replacement, and gene disruption in *Chlamydia*. We believe the ability to stably transform *C. psittaci* 6BC to antibiotic resistance using a synthetic version of the 16S rRNA gene represents a significant technical advance in the study of *Chlamydia* as it lays the groundwork to develop methods that integrate foreign DNA in these genetically intractable pathogens. The next step will be the production of mutants and marked strains of *Chlamydia* to address fundamental questions of *Chlamydia* genetics, biology, and pathogenesis.

Materials and Methods

Bacterial Strains. The bacterial strains are listed in Table S1. *E. coli*, *C. psittaci* serovar 6BC, and *C. trachomatis* serovar L2/LGV/434/Bu were grown as previously described (9).

Titration, Antimicrobial Susceptibility Testing, and Isolation of *C. psittaci* 6BC Spontaneous Mutants. The susceptibilities of *C. psittaci* 6BC to antibiotics were determined in the plaque assay as previously described (9, 10). Ksm (BIOMOL) was diluted in 2× DMEM (GIBCO) to a final concentration of 10 mg/mL and stored at 4 °C. Other aminoglycosides were purchased from Sigma Chemical Co. and diluted in 2× DMEM, with the exception of Spc. Kanamycin A, kanamycin B, hygromycin B, streptomycin, apramycin, tobramycin, and paromomycin, were added in the plaque assay to final concentrations of 1 mg/mL, 1 mg/mL, 1 mg/mL, 8 mg/mL, 2 mg/mL, 1.5 mg/mL, and 2 mg/mL, respectively. Higher concentrations were toxic to the tissue culture cells. Isolation and analysis of *C. psittaci* spontaneous KSM^R variants was done as previously described (9, 10).

General DNA Manipulations. All DNA manipulations were done using standard procedures (25). Details on the construction of the different plasmids are included in Table S1, and PCR primers are listed in Table S2. DNA sequences were aligned using Clone Manager 8 (Scientific and Educational Software).

Electroporation of *C. psittaci* 6BC and *C. trachomatis* L2. Highly purified preparations of EBs, obtained by centrifugation through RenoCal-76 (Bracco Diagnostics) density gradients, were washed once with sterile cold dH_2O, diluted in dH_2O, and stored on ice for up to 2 h. Alternatively, crude preparations of EBs were washed 4 times with dH_2O before electroporation. Between 0.5 and 50 μg of DNA, either as supercoiled circular plasmid or after NotI-digestion to separate the rRNA region from the vector backbone, was added to 10^7–10^8 PFUs, transferred to cold 0.1-cm electroporation cuvettes, and electroporated using a Gene Pulser (Bio-Rad) under various voltage and resistance settings, but with the capacitance fixed to 25 μF (Table 2). DNA for electroporation into *Chlamydia* spp. was extracted from different *E. coli* strains (Table S1), following the QIAfilter Plasmid Midi or Maxi Kit procedure (QIAGEN), and further purified with phenol and chloroform before ethanol precipitation. Immediately after electroporation, the bacteria were collected in 450 μL of various cold buffers depending on the applications: SPG (250 mM sucrose, 10 mM sodium phosphate, 5 mM L-glutamic acid) for storage at −80 °C; 1× PBS with 10 mM $MgCl_2$ for the DNase I digestion (Invitrogen); and 1× DMEM to inoculate L2 mouse fibroblast cells in the plaque assay (9).

Comparative qPCR. *C. psittaci* 6BC and *C. trachomatis* L2 were electroporated with 500 ng of a pUC derivative carrying the foreign marker *aadA* and resuspended in 150 μL 1× PBS containing 10 mM $MgCl_2$. Four microliters was saved in SPG to determine the titer in the plaque assay. The remaining volume was incubated 4 successive times with 146 units of DNase I for 15 min at 37 °C. The digestion was then stopped with 3 mM EDTA. Total DNA was collected after RNase A treatment in 200 μL of elution buffer with the DNeasy Tissue Kit (QIAGEN) and stored at 4 °C. PCR analysis using primers (Table S2) designed to amplify part of the bacterial *folA* gene showed that the bacterial chromosome was protected from the DNase as expected (data not shown), whereas primers specific to the vector *aadA* gene revealed that part of the recombinant plasmid was protected from the DNase after electroporation (data not shown). Real-time qPCR (12) that is based on the number of cycles needed for amplification-generated fluorescence to reach a specific threshold of detection (the Ct value) allowed comparison of the amount of vector detected after electroporation to the background level detected in the absence of electroporation. Primers were designed to amplify the 16S rRNA gene from *C. psittaci* 6BC and *C. trachomatis* L2, serving as an endogenous reference gene, and *aadA* from the transforming vector (Table S2). Melting curve analysis showed that the accumulation of SYBR green-bound DNA was gene specific and not due to primer dimers. Data were analyzed by the $2^{-\Delta\Delta CT}$ method (26).

Southern Blot. Southern blot analysis of transforming DNA sequences in *C. psittaci* 6BC was performed on total DNA collected from infected cells (Figs. S2 and S3*B*) using DNeasy Tissue Kits (QIAGEN) or on genomic DNA prepared using the same kits from highly purified preparations of EBs obtained by centrifugation through RenoCal-76 density gradients (Fig. 2 *B* and *C* and Fig. S3*A*). Aliquots of DNA were digested with SacII or HpaI overnight and analyzed by Southern gel transfer following standard techniques (25). Blots were probed with a nonradioactive, digoxigenin-11-dUTP-labeled probe (Roche Molecular Biochemicals); a 300- or 509-bp DNA fragment internal to the *C. psittaci* 6BC 16S gene amplified by PCR using, respectively, the RT2 and 16S8 primers (Figs. 1 and 2*B* and Fig. S3) or the 16S1 and RT1 primers (Table S2); or a 370-bp nonradioactive fragment internal to the vector *cat* gene generated by PCR amplification using the cat-F and the catM-R primers (Table S2 and Fig. 2*C*). Hybridization and immunological detection of the probe were performed as described by Panaud *et al.* (27) using the Thermo Scientific Pierce SuperSignal West Pico Chemiluminescent Substrate (Fisher Scientific) according to the manufacturer's directions.

Additional Methods. Details on the purification of *C. trachomatis* L2 cryptic plasmid and on the experimental steps leading to purification of *C. psittaci* 6BC recombinants can be found in *SI Text*.

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